



Docket No.: 31075/40037  
(PATENT)

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Application of: Zankel *et al*

Application No.: 10/812,849

Group Art Unit: 1649

Filing Date: March 30, 2004

Examiner: D. Kolker

For: Megalin Based Delivery of Therapeutic  
Compounds to the Brain and Other Tissues

**DECLARATION UNDER 37 C.F.R. § 1.132 OF DR. TODD ZANKEL**

Commissioner for Patents  
P.O. Box 1450  
Alexandria, Virginia 22313-1450

Dear Sir:

I, Dr. Todd Zankel, do hereby declare and state as follows:

1. I am a co-inventor of the invention claimed in the above-referenced application.
2. The following experiments were carried out at my direction and under my supervision. The construction of a RAP fragment as described was readily accomplished with only routine effort.
3. A C-terminal fragment of RAP starting at amino acid 201 of mature RAP and lacking the HNEL endoplasmic reticulum retention signal at the C-terminus, was prepared. Briefly, cDNA encoding this fragment was ligated into Novagen vector pET30+, the vector was expressed in E. coli BL21(DE3) cells, and the RAP fragment was purified generally as described in WO 2006/138343, an application on which I am a named inventor. This RAP d3 fragment consisted of amino acids 201-319 of SEQ ID NO: 1.

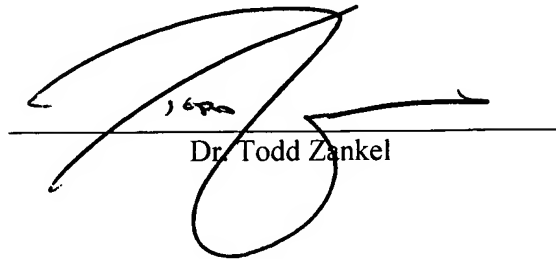
4. Binding of the RAP C-terminal fragment (called "RAP d3") to megalin receptor expressed on human primary renal proximal tubule epithelial cells (RPTEC, Cambrex) was assayed as follows. RPTEC expresses megalin and not LRP1 (*J. Biol. Chem.* 267:29, 21162-21166, 1992). <sup>125</sup>I-labeled RAP d3 was incubated with cells at 50 nM in triplicate in 12-well plates in phosphate-buffered saline (PBS) containing calcium and magnesium and either 500 nM RAP d3 or 500 nM full-length RAP (amino acids 1-319 of mature RAP). Binding was performed on ice for 1 hour followed by three washes with cold buffer. Washed cells were lysed in 2N NaOH and radioactivity quantitated with a gamma-counter. Results are displayed in Figure 1 attached, where binding of RAP d3 to the cells in the presence of PBS (control) is shown as "RPTEC", binding of RAP d3 to the cells in the presence of RAP is shown as "RAP", and binding of RAP d3 to the cells in the presence of RAP d3 is shown as "RAP d3". RAP d3 showed specific binding to megalin as shown by efficient competition for binding with either RAP d3 or full-length RAP on these cells. Inhibition of RAP d3 binding by excess cold RAP d3 or excess cold full-length RAP did not differ significantly.

5. In order to determine the affinity of RAP d3 for sites on megalin, the binding of RAP d3 to CHO-K1 LRP-deficient cells expressing a megalin mini-receptor consisting of ligand-binding domain 2, the transmembrane domain and the cytoplasmic domain (megalin LB2) was measured. Cells were seeded in 12-well plates and grown to confluence. <sup>125</sup>I-labeled RAP d3 was incubated at various concentrations in triplicate with the cells on ice in phosphate-buffered saline containing calcium and magnesium for 1 hour. Cells were then washed extensively with cold buffer, lysed in 2N NaOH and counted with a gamma counter. The binding isotherm was fitted by non-linear regression with the assumption of a single binding site. The calculated dissociation constant for the complex of RAP d3 and the megalin mini-receptor was  $12 \pm 2$  nM, similar to previously reported values for the complex of full-length RAP with megalin. Results are displayed in Figure 2 attached.

6. The data described above demonstrate that a C-terminal fragment of RAP consisting of amino acids 201-319 bound specifically to megalin, with an affinity equivalent to full-length RAP.

7. I further declare that all statements made herein of my own knowledge are true, that all statements made on information and belief are believed to be true, and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both (18 U.S.C. § 1001), and may jeopardize the validity of the application or any patent issuing thereon.

Date 2/21/07



Dr. Todd Zankel